

MEMBRANE POTENTIAL, CHLORIDE EXCHANGE, AND CHLORIDE CONDUCTANCE IN EHRLICH MOUSE ASCITES TUMOUR CELLS

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SUMMARY

1. The steady-state tracer exchange flux of chloride was measured at 10–150 mM external chloride concentration, substituting either lactate or sucrose for chloride. The chloride flux saturates in both cases with a $K_{\frac{1}{2}}$ about 50 and 15 mM, respectively.

2. The inhibitory effect of other monovalent anions on the chloride transport was investigated by measuring the $^{36}\text{Cl}^-$ efflux into media where either bromide, nitrate, or thiocyanate had been substituted for part of the chloride. The sequence of increasing affinity for the chloride transport system was found to be: $\text{Br}^- < \text{Cl}^- < \text{SCN}^- = \text{NO}_3^-$.

3. The chloride steady-state exchange flux in the presence of nitrate can be described by Michaelis–Menten kinetics with nitrate as a competitive inhibitor of the chloride flux.

4. The apparent activation energy (E_A) was determined to be 67 ± 6.2 kJ/mole, and was constant between 7 and 38 °C.

5. The membrane potential (V_m) was measured as a function of the concentration of external K^+ , substituting K^+ for Na^+ . The transference number for K^+ (t_K) was estimated from the slope of V_m vs. $\log_{10} (\text{K}^+)_e$, and t_{Cl} and t_{Na} were calculated, neglecting current carried by ions other than Cl^- , K^+ , and Na^+ . The diffusional net flux of K^+ was calculated from the steady-state exchange flux of $^{42}\text{K}^+$, assuming the flux ratio equation to be valid. From this value the K^+ conductance and the Na^+ and Cl^- conductances were calculated. The experiments showed that G_{Cl} , G_{Na} , and G_K are all about $14 \mu\text{S}/\text{cm}^2$.

6. The net (conductive) chloride permeability derived from the chloride conductance was 4×10^{-8} cm/sec compared with the apparent permeability of 6×10^{-7} cm/sec as calculated from the chloride tracer exchange flux. These data suggest that about 95% of the chloride transport is mediated by an electrically silent exchange diffusion.

7. Comparable effects of phloretin (0.25 mM) on the net (conductive) permeability and the apparent permeability to chloride (about 80% inhibition) may indicate that the chloride exchange and conductance pathways are not completely separate and

Experiments were performed at both addresses.

distinct modes of transport, but may involve common elements. The reduced chloride permeability in the presence of phloretin is estimated to be two orders of magnitude larger than the ground permeability of the cell membrane.

INTRODUCTION

It has been generally assumed that chloride transport in Ehrlich ascites tumour cells is caused by a simple diffusion process (Grobeck, Kromphardt, Mariani & Heinz, 1963; Hempling & Kromphardt, 1965; Kromphardt, 1968; Aull, 1972). Recently, however, Levinson & Villereal (1976) have demonstrated that a part of the chloride flux is caused by mediated diffusion and evidence has been presented that the major part of the chloride movement represents an exchange diffusion component (Heinz, Geck & Pietrzyk, 1975; Heinz, Geck, Pietrzyk, Burckhardt & Pfeiffer, 1977; Aull, Nachbar & Oppenheim, 1977).

This study shows that the chloride steady-state tracer exchange flux is saturable, is inhibited by other monovalent anions, and has a relatively large temperature dependence. Furthermore, the chloride conductance as estimated from micro-electrode measurements of the membrane potential at varying external K^+ concentration corresponds only to about 6% of the value expected from the tracer exchange flux. These results strongly suggest that the chloride transport is mediated predominantly by an exchange diffusion process as defined by Ussing (1947). In addition, both chloride exchange and conductance are inhibited to about the same extent by phloretin, suggesting that a common element may be involved in the chloride conductance and the electrically silent exchange diffusion. Preliminary reports on this work have been published (Hoffmann, Simonsen & Sjøholm, 1975; Simonsen, Hoffmann & Sjøholm, 1976; Sjøholm, Hoffmann & Simonsen, 1976).

METHODS

Cell suspensions

Ehrlich mouse ascites tumour cells (hyperdiploid strain) were maintained by weekly intraperitoneal transplantation in white female NMRI mice and 8 days after transplantation harvested in a basic Ringer solution containing heparin (2.5 i.u./ml.). This solution had the following composition (mM): Na^+ , 150; K^+ , 5; Mg^{2+} , 1; Ca^{2+} , 1; Cl^- , 150; sulphate, 1; inorganic phosphate, 1; MOPS (morpholinopropane sulphonic acid), 3.3; TES (*N*-tris-(hydroxymethyl)methyl-2-amino-ethane sulphonic acid, 3.3; and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2 ethane-sulphonic acid), 5; pH 7.40 (cf. Eagle, 1971). The cells were washed by centrifugation (45 sec, $700 \times g$) first with the basic Ringer solution and then with the appropriate incubation medium (see below), and the cytocrit adjusted to 8%. The cell suspensions were incubated at the experimental temperature in a shaking bath for 20–110 min before the flux experiments were carried out, with the longest pre-incubation in experiments at lower temperatures. [3H] inulin (The Radiochemical Centre, Amersham, England, 5×10^6 dpm/ml.) was added as marker of extra-cellular space (Hendil & Hoffmann, 1974). The pH of all solutions was 7.40 ± 0.05 during incubation.

In the incubation media, the Cl^- concentration of the basic Ringer solution was varied from 5 to 150 mM either by equimolar substitution of NaBr, $NaNO_3$, NaSCN, or Na L-lactate for NaCl or by substitution of sucrose for NaCl. If isosmotic sucrose is substituted for NaCl the cells will shrink due to a loss of osmotically active substances (Hendil & Hoffmann, 1974). In preliminary experiments, however, substitution of 0.85 m-mole sucrose per m-mole NaCl was found to give constant cell volume (measured in a Coulter Counter, model ZB with Channelyzer

C-1000, Coulter Electrics Ltd., England). In other experiments the K⁺ concentration of the basic Ringer solution was varied from 5 to 150 mM by equimolar substitution of K⁺ for Na⁺. Phloretin (K & K Laboratories, Inc., California) was added from a 0.25 M stock solution in ethanol.

Measurement of ion concentrations

Quadruplicate 500 μ l. samples of the cell suspensions were transferred to preweighed vials for determination of ion content and cell water. The vials were centrifuged ($20\,000 \times g$, 60 sec), the supernatant removed by suction and the samples weighed. In two of the samples cell water was determined by drying for 48 hr at 90 °C and reweighing. In the two other samples the packed cells were lysed in 400 μ l. distilled water and deproteinized with addition of 50 μ l. perchloric acid (70 %). Two 50 μ l. samples of the supernatant were saved and processed in parallel with the cell pellets for determination of ion content.

K⁺ and Na⁺ were determined by emission flame photometry (Pye-Unicam SP90B Atomic Absorption Photometer, Pye-Unicam, Ltd., England) after dilution of samples and standards with 1 mM-CsCl in order to increase the linearity of standard curves and to eliminate the interference of Na⁺ in K⁺-measurements and *vice versa*. Cl⁻ concentration was assessed by coulometric titration (CMT 10 Chloride Titrator, Radiometer, Copenhagen, Denmark). In some experiments the concentration of Br⁻ or SCN⁻ was assessed by coulometric titration of the sum of Cl⁻ and the respective anion and subtraction of the known Cl⁻ concentration. In these experiments the cell chloride was calculated from the known extracellular Cl⁻ concentration and the measured distribution ratio of ³⁶Cl⁻.

³H, ³⁶Cl⁻, and ⁴²K⁺ activities were measured in a liquid scintillation spectrometer (Packard Tri-Carb 3375) using 1 ml. diluted sample in 10 ml. scintillation liquid (5.5 g Permablend[®] III (Packard) and 500 ml. Triton X-100 (Rohm & Haas) per litre toluene (BDH), cf. Turner, 1969). The separation of ³H and ³⁶Cl⁻ counts was optimized using the method of Bush (1964). The cellular concentrations are given as the concentration in cell water after correction for trapped volume ([³H] inulin space) in the cell pellets.

³⁶Cl⁻ and ⁴²K⁺ flux experiments

The chloride steady-state exchange flux was measured as influx (as described by Simonsen & Nielsen (1971)), or as efflux. In efflux experiments the cell suspensions were equilibrated with ³⁶Cl⁻ (Riso, Denmark; 10⁶ dpm/ml.) at the experimental temperature for a period greater than six half-times of the chloride exchange. In experiments carried out below 17 °C, however, the cells were incubated with the isotope at 38 °C for 15 min followed by at least 45 min at the experimental temperature. Duplicate samples of labelled cells for transference to the efflux medium were obtained by centrifugation of 1 ml. cell suspension in nylon tubes (i.d. 3 mm) for 1 min at $770 \times g$, and cutting the tube 1 mm below the interface between the packed cells and the medium. The packed, labelled cells (about 160 mg) were then injected into 35 ml. of the appropriate medium by flushing the small sleeve of nylon tube containing the packed cells with 5 ml. of the medium. The efflux of ³⁶Cl⁻ from the cells into the medium was then followed with time by serially isolating cell-free medium by rapid filtration of the cell suspension as described by Dalmark & Wieth (1972), but with addition of an extra prefilter (Millipore AP10). In the ⁴²K⁺ influx experiments the rise in radioactivity in the cellular compartment was followed with time as described by Hendil & Hoffmann (1974).

Calculations of tracer exchange fluxes. The tracer exchange experiments were performed under steady-state conditions and in the individual experiments the kinetics were well described by a closed two compartment model with constant volumes. The time dependence of the specific activity of ³⁶Cl⁻ or ⁴²K⁺ in the cells or in the medium is described by the following equations:

$$a_t - a_o = (a_{\infty} - a_o) \times (1 - e^{-\lambda t}) \quad (1)$$

$$\lambda = k(1 + Q_o/Q_e) \quad (2)$$

where a_t , a_o , and a_{∞} are the specific activities at time t , at zero time, and at isotope equilibrium, respectively; λ is the observed rate constant, and k the true rate constant of cellular exchange; Q_o and Q_e represent the total amount of chloride or K⁺ in the intra- and extracellular compartment, respectively.

The exponential constant (λ) was determined by an iterative non-linear unweighted least-squares analysis of the flux curves. The rate constant of cellular exchange (k) equals λ when the extracellular compartment (Q_e) can be regarded as infinite relative to the intracellular compartment (Q_i). In the $^{36}\text{Cl}^-$ efflux experiments this condition was fulfilled because the cytocrit was below 0.5 %. In the influx experiments, however, where the cytocrit was 8 %, k was calculated according to eqn. (2). This gave a very small correction in the $^{36}\text{Cl}^-$ experiments, but an important correction in the $^{42}\text{K}^+$ experiments.

The steady-state tracer exchange flux of chloride or K^+ (J) was obtained from the

$$J = k \times (C)_i \times (\text{H}_2\text{O})_i \text{ (}\mu\text{mole/g dry wt.} \times \text{min)}$$

where $(C)_i$ is the Cl^- or K^+ concentration in cell water, and $(\text{H}_2\text{O})_i$ is the cell water content (g/g cell dry wt.). The fluxes were converted from units of $\mu\text{mole/g dry wt.} \times \text{min}$ to $\text{p-mole/cm}^2\text{.sec}$ by multiplication by the factor 0.818 (1 g cell solids corresponds to 2.0×10^9 cells with a total surface area of $2.0 \times 10^4 \text{ cm}^2$, assuming a cell density of 1.06 g/ml. (Hendil & Hoffmann, 1974), a dry to wet weight ratio of 0.24, and that a single cell has a volume of $1.93 \times 10^{-9} \text{ ml.}$ and a surface area of $1.0 \times 10^{-8} \text{ cm}^2$ (Lassen, Nielsen, Pape & Simonsen, 1971)).

Potential measurements

An aliquot of Ehrlich cells incubated in basic Ringer solution was washed and resuspended in the experimental medium at a cytocrit of 0.1–0.5 % and then immediately introduced into the micro-electrode measuring chamber. The membrane potential was measured directly by penetration of the cell membrane with a conventional KCl (2.5 M) filled micro-electrode with a tip diameter of about 0.2 μm . The cell was impaled by rapid advancement of the micro-electrode by aid of a Piezo-electric electro-mechanical transducer as previously described (Lassen *et al.* 1971). Usually potential recordings from ten to twenty cells were obtained 2–12 min after the change in suspending medium. The peak negative potential recorded within 0.2 msec after penetration of the cell membrane with the micro-electrode tip is used as a measure of the membrane potential of the unperturbed cell. The subsequent decay of the recorded potential towards a less negative value is ascribed to mechanical damage of the cell membrane, and dissipation of the membrane potential through an electrical leak around the impaling micro-electrode. This interpretation has been discussed by Lassen (1977) and Lassen & Rasmussen (1978) but has recently been questioned by Maloff, Scordilis, Reynolds & Tedeschi (1978).

For technical reasons we could not record the membrane potential earlier than about 2 min after the change in external medium. Thus, it was essential to ascertain whether or not there had been any drift in membrane potential before the recordings took place. An estimate of the net ion movements and resulting change in membrane potential within the first 2 min after an increase in external (K^+) from 5 to 10 mM, or exposure to phloretin, or both, was obtained in calculations using the Goldman–Hodgkin–Katz equations for net flux and membrane potential (Hodgkin & Katz, 1949). Inserting the measured membrane potentials and the membrane permeabilities to K^+ , Na^+ , and Cl^- (see Table 4) the calculated changes in cellular ion concentrations were about 1 mM or less, and the expected change in membrane potential less than 0.5 mV. Furthermore, in a control experiment external (K^+) was increased from 5 to 17 mM and the cellular ion concentrations followed with time. The cellular concentrations were found to change by less than 12 mM in 35 min, and the expected change in membrane potential was less than 0.4 mV. The membrane potentials recorded during the period from 2 to 12 min after the change in external medium showed only a slight decrease with time. The above results are in agreement with the findings in muscle fibers (Hodgkin & Horowicz, 1959), where potential recordings could be made immediately after a shift in external K^+ concentration. In conclusion, the drift in membrane potential before the first potential recordings were made, appears to be negligible. Substituting K^+ for external Na^+ also leads to a slow cell swelling. At 125 mM-external K^+ this amounts to 7–8 % after 12 min and a slight effect on the membrane permeabilities is to be expected (Hoffmann, 1978).

Calculation of membrane conductance, conductive unidirectional flux, and membrane permeability

The membrane conductance for K⁺ (G_K) was calculated from the steady-state exchange flux of ⁴²K⁺. The flux ratio equation (Ussing, 1949), was assumed to be valid for the unidirectional K⁺-efflux (J_K^{lo}) and the passive component of the K⁺-influx ($J_K^{ol(d)}$):

$$J_K^{lo}/J_K^{ol(d)} = e^{(V_m - E_K)F/RT} \quad (3)$$

V_m is the membrane potential, E_K is the Nernst potential for K⁺, and F , R and T are Faraday's constant, the gas constant, and the absolute temperature, respectively. From the calculated value of $J_K^{ol(d)}$, and hence the diffusional netflux of K⁺ ($J_K^{net(d)} = J_K^{ol(d)} - J_K^{lo}$), G_K could be calculated from the equation

$$G_K = F \times J_K^{net(d)} / (E_K - V_m) = F(J_K^{ol(d)} - J_K^{lo}) / (E_K - V_m) \quad (4)$$

Since chloride is assumed to be in electrochemical equilibrium across the cell membrane, the conductive (electro-diffusional) unidirectional flux ($J_{Cl}^{lo(d)} = J_{Cl}^{ol(d)}$) corresponding to the chloride conductance (G_{Cl}) could be calculated from the equation (Ussing & Zerahn, 1951; Hodgkin, 1951):

$$G_{Cl} = \frac{F^2}{RT} \times J_{Cl}^{lo(d)} \quad (5)$$

From $J_{Cl}^{lo(d)}$ the net (conductive) membrane permeability to chloride (P_{Cl}) was calculated, assuming a linear electrical potential profile and constant ionic mobilities throughout the membrane (Hodgkin & Katz, 1949; see Sten-Knudsen, 1978):

$$J_{Cl}^{lo(d)} = P_{Cl} \times f_{V_m}^{lo} \times (Cl^-)_o = P_{Cl} \times \frac{(-V_m)F/RT}{1 - e^{V_m F/RT}} \times (Cl^-)_o \quad (6)$$

where $f_{V_m}^{lo}$ is the factor representing the effect of the electrical field on the unidirectional efflux (cf. Katz, 1966). The apparent P_{Cl} , i.e. the permeability constant corresponding to a conductive, unidirectional flux of the same magnitude as the measured chloride steady-state tracer exchange flux, was also calculated using eqn. (6).

In the presence of phloretin eqn. (5) cannot be applied, V_m being significantly more negative than the Nernst potential for chloride (E_{Cl}) (see Table 3). Therefore, the diffusional net flux of Cl⁻ was calculated from G_{Cl} (cf. eqn. (4)). From this value and the flux ratio equation (cf. eqn. (3)) $J_{Cl}^{lo(d)}$ was calculated, and hence P_{Cl} from eqn. (6). P_{K_s} was calculated following a similar procedure, and P_K was calculated from the unidirectional K⁺ efflux (J_K^{lo}) determined as the steady-state exchange flux of ⁴²K⁺ (cf. eqn. (6)).

Statistical evaluation. All values are expressed as the mean \pm s.e. of mean with the number of experiments in brackets. Student's *t* test was used to evaluate statistical significance.

RESULTS

Typical ³⁶Cl⁻ efflux and influx experiments are shown in Fig. 1. The two types of experiments gave nearly identical values for the chloride steady-state exchange flux. The ratio between the means of three influx and seven efflux experiments from the same series was 0.92 ± 0.15 . In most experiments only ³⁶Cl⁻ efflux was measured. The mean value for all the efflux experiments was 63 ± 4 p-mole/cm². sec ($n = 19$), similar to the value of 65 p-mole/cm². sec published earlier for influx measurements (Lassen *et al.* 1971).

Saturation of the chloride transport

The steady-state exchange flux of chloride was measured in 10–150 mM-external (Fig. 2A) or 6–76 mM-internal Cl⁻ concentration (Fig. 2B), substituting equimolar concentrations of lactate for Cl⁻. Before starting the efflux measurements the cells

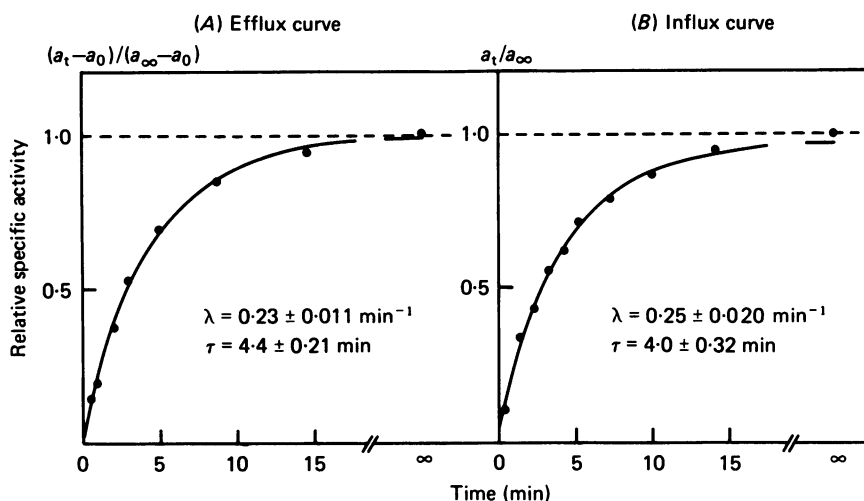


Fig. 1. $^{36}\text{Cl}^-$ efflux and $^{36}\text{Cl}^-$ influx curves in basic Ringer solution at 38°C . The relative specific activity (i.e. the specific activity at time t (a_t) expressed as a fraction of the specific activity of the medium at isotopic equilibrium (a_∞)) was followed with time in medium (A) or in cells (B). The single exponential function with the best fit was calculated as described in Methods. λ is the observed rate constant and τ ($= 1/\lambda$) is the time constant. In the efflux curve (A) the $^{36}\text{Cl}^-$ activity transferred as extracellular $^{36}\text{Cl}^-$ at zero time (a_0) is subtracted, the amount being assessed with [^3H]inulin as extracellular marker.

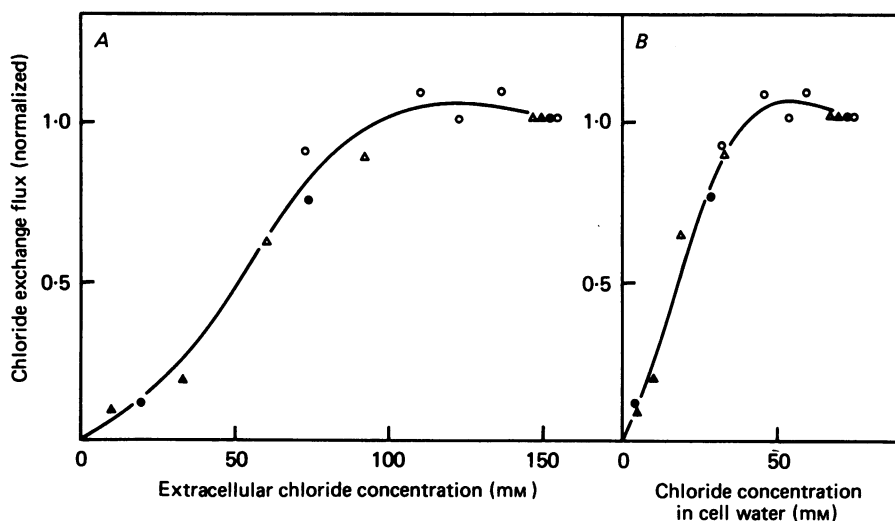


Fig. 2. Chloride steady-state exchange flux at 38°C as a function of extracellular (A) or intracellular (B) chloride concentration. The chloride concentration in the medium was varied by substituting lactate for chloride, keeping the sum constant at 150 mM. The cell volume was increased by $14 \pm 3\%$ ($n = 5$) of the normal volume when 100 mM-lactate was substituted for chloride. The chloride distribution ratio between cell water and medium (r_{Cl}) was decreased with increasing lactate concentration, from 0.55 to 0.33. The fluxes are given as fraction of the flux (control) obtained at 150 mM-extracellular chloride ($63 \pm 4 \text{ p-mole/cm}^2 \cdot \text{sec}$ ($n = 19$), mean value \pm s.e. of mean). The measurements are from four separate experiments as indicated by the different symbols.

were equilibrated for 45 min with the experimental medium until a steady-state was achieved with respect to both anion and cation distribution. It can be seen that the chloride transport mechanism saturates above 90 mM-external or 40 mM-internal Cl⁻ concentration with a $K_{\frac{1}{2}}$ of approx. 50 mM in the medium or 20 mM in the cell water.

In other experiments the Cl⁻ concentration was varied by substituting sucrose for extracellular NaCl (see Methods). Figs. 3A and B show the chloride steady-state

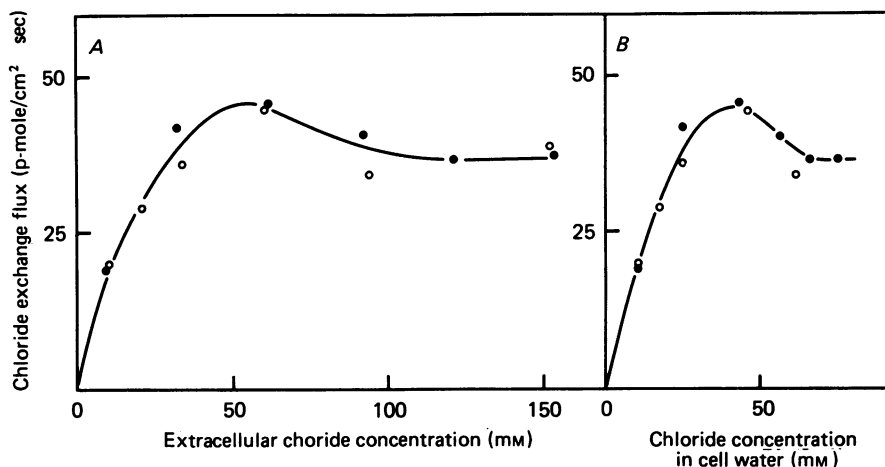


Fig. 3. Chloride steady-state exchange flux at 38 °C as a function of extracellular (A) or intracellular (B) chloride concentration. The chloride concentration in the medium was varied by substituting sucrose for sodium chloride (see Methods). The cell volume was constant. The chloride distribution ratio between cell water and medium (r_{Cl}) was increased with increasing sucrose concentration from 0.6 to 1.1. The measurements are from two separate experiments as indicated by the different symbols. Other experiments gave similar results, but were quantitatively slightly different.

exchange flux measured in 10–150 mM-external or 10–80 mM-internal Cl⁻ concentration. Again, the cells were pre-equilibrated with the experimental medium for 45 min. It can be seen that the chloride flux reaches a maximum at 50 mM extracellular or 40 mM-intracellular Cl⁻ concentration and decreases slightly at higher Cl⁻ concentrations. $K_{\frac{1}{2}}$ is approx. 15 mM in the medium and 13 mM in the cell water.

Effect of other monovalent anions on chloride transport

The ³⁶Cl⁻ efflux was measured into media where either Br⁻, SCN⁻, or NO₃⁻ had been substituted for about half of the Cl⁻, the cells being equilibrated for 45–90 min and labelled with ³⁶Cl⁻ in the appropriate media before starting the efflux experiments. All experiments were performed under steady-state conditions with respect to anion concentrations. The Na⁺ concentration in cell water was within the range 30–35 mM and the K⁺ concentration within the range 181–196 mM. The results are shown in Table 1.

In Br⁻ medium the rate constant was unchanged or slightly increased compared to the control. The chloride exchange flux in Br⁻ medium was reduced to 0.60 times the control value ($P < 0.01$), compared to a reduction to 0.85 at a comparable Cl⁻

TABLE 1. The effect of anion substitution on the chloride exchange flux in Ehrlich ascites cells. The respective monovalent anion replaces chloride, keeping the total constant at 150 mM in the extracellular medium. E_{Cl} is the Nernst potential for Cl^- , $(\text{H}_2\text{O})_0$ the cell water, and k the rate constant of cellular Cl^- exchange. J_{Cl} is the steady-state exchange flux of chloride. The values are given as mean \pm s.e. of mean, n is the number of independent experiments

Substituting anion	(n)	$(\text{Cl}^-)_0$ (mM)	$(\text{Cl}^-)_i$ (mM)	E_{Cl} (mV)	$(\text{H}_2\text{O})_0$ (g/g dry wt.)	k (min^{-1})	J_{Cl} (p-mole/ $\text{cm}^2 \cdot \text{sec}$)
Control	(5)	151 ± 1.1	74 ± 2.3	-19 ± 0.9	3.6 ± 0.06	0.32 ± 0.025	68 ± 4.2
Bromide	(5)	79 ± 0.9	38 ± 1.4	-20 ± 0.9	3.6 ± 0.10	0.38 ± 0.046	41 ± 2.9
Thiocyanate	(5)	80 ± 0.7	38 ± 1.4	-20 ± 0.8	3.3 ± 0.06	0.26 ± 0.032	25 ± 3.2
Nitrate	(4)	80 ± 0.7	39 ± 1.6	-19 ± 1.1	3.5 ± 0.15	0.22 ± 0.038	26 ± 3.3

concentration in lactate medium (Fig. 2). Thus, Br^- seems to be an inhibitor of the Cl^- transport.

The chloride exchange flux in Br^- medium was significantly higher than the flux in SCN^- medium ($P < 0.01$) and in NO_3^- medium ($P < 0.01$). Thus, SCN^- and NO_3^- have a significantly higher inhibitory potency than Br^- on the chloride flux. The cell volume was unchanged in the various media (Table 1, column 6). Furthermore, the distribution ratios for the substituting anions Br^- and SCN^- were equal to the distribution ratio for Cl^- , and the calculated E_{Cl} was virtually identical in the different media (Table 1, column 5), indicating an unchanged membrane potential. Thus, if the membrane potential contributes to the driving force for tracer efflux, the effect should be equal in all media and cannot be responsible for the differing rates of chloride exchange.

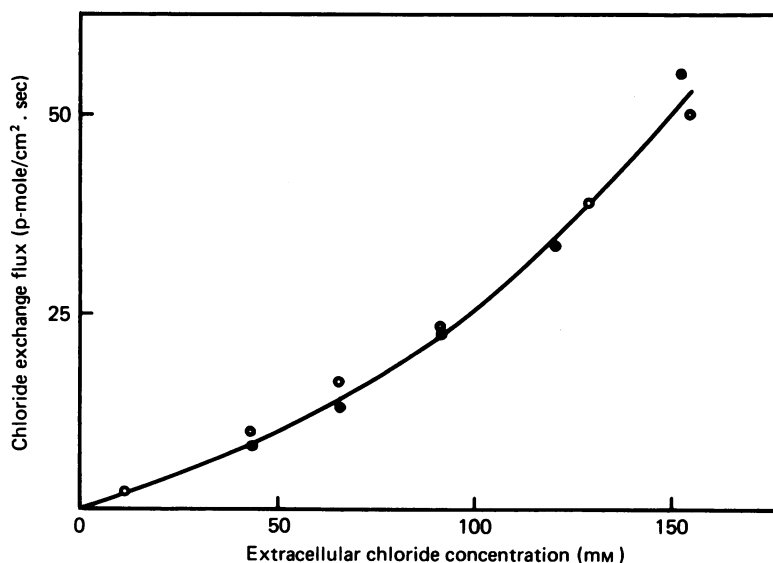


Fig. 4. Chloride steady-state exchange flux at 38 °C as a function of extracellular chloride concentration. Nitrate was substituted for chloride in equimolar amounts, the sum being kept constant at 150 mM. The cell volume and r_{Cl} were constant. The measurements are from two separate experiments as indicated. Other experiments gave similar results, but were quantitatively slightly different.

Chloride exchange in nitrate media

The chloride steady-state exchange flux was found to be progressively inhibited following substitution of increasing amounts of NO₃⁻ for Cl⁻ (Fig. 4).

If the saturation kinetics for chloride can be treated as a Michaelis-Menten type of

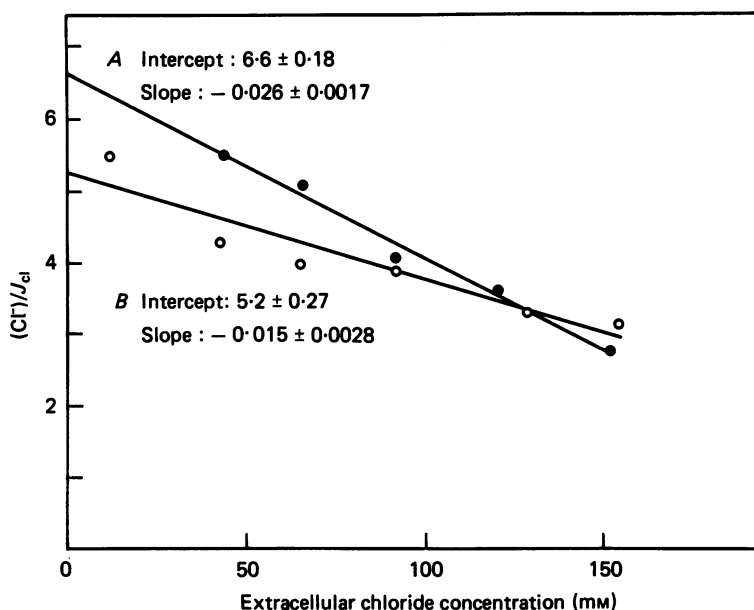


Fig. 5. A plot of $(\text{Cl}^-)/J_{\text{Cl}}$ vs. (Cl^-) in the extracellular medium (cf. eqn. (8)). J_{Cl} is the chloride steady-state exchange flux (expressed as p-mole/cm².sec and (Cl^-) the extracellular chloride concentration (in mM). Data from two separate experiments (A, B) (same data as in Fig. 4). The straight lines indicate a competitive inhibition, whereas non-competitive inhibition would give a parabolic curve (convex upwards).

kinetics, then for a *competitive* inhibitor (I), and $(\text{Cl}^-) + (\text{I}) = \text{H}$, one would find (cf. Gunn, Dalmark, Tosteson & Wieth, 1973):

$$J_{\text{Cl}} = \frac{J_{\text{Cl}}^{\text{max}}}{1 + \frac{K_m}{(\text{Cl}^-)} \left(1 + \frac{(\text{I})}{K_i}\right)} \quad (7)$$

where J_{Cl} is the unidirectional steady-state exchange flux for chloride, $J_{\text{Cl}}^{\text{max}}$ is the maximal J_{Cl} , (Cl^-) the Cl⁻ concentration, K_m the Michaelis-constant, K_i the inhibitory constant, and H is the total anion concentration, which is kept constant. By combination and rearrangement it can be seen that $(\text{Cl}^-)/J_{\text{Cl}}$ should be linearly dependent on (Cl^-) :

$$\frac{(\text{Cl}^-)}{J_{\text{Cl}}} = \frac{1}{J_{\text{Cl}}^{\text{max}}} \times \left[\left(1 - \frac{K_m}{K_i}\right) \times (\text{Cl}^-) + K_m \left(1 + \frac{\text{H}}{K_i}\right) \right] \quad (8)$$

In contrast, for a *non-competitive* inhibitor, where $(\text{Cl}^-) + (\text{I}) = \text{H}$, $(\text{Cl}^-)/J_{\text{Cl}}$ should be dependent on $(\text{Cl}^-)^2$ as seen from the following equations:

$$J_{\text{Cl}} = \frac{J_{\text{Cl}}^{\text{max}}/(1 + (I)/K_1)}{1 + \frac{K_m}{(\text{Cl}^-)}} \quad (9)$$

$$\frac{(\text{Cl}^-)}{J_{\text{Cl}}} = \frac{1}{J_{\text{Cl}}^{\text{max}}} \times \left[-\frac{(\text{Cl}^-)^2}{K_1} + \left(1 - \frac{K_m}{K_1} + \frac{H}{K_1}\right)(\text{Cl}^-) + K_m \left(1 + \frac{H}{K_1}\right) \right] \quad (10)$$

Fig. 5 shows that $(\text{Cl}^-)/J_{\text{Cl}}$ is linearly dependent on (Cl^-) . Thus, nitrate is likely to be a competitive inhibitor of the chloride exchange flux. The negative slope indicates that the affinity for nitrate is higher than for chloride ($K_1 < K_m$, cf. eqn. (8)), consistent with the results shown in Table 1.

TABLE 2. The inhibitory effect of other monovalent anions on the chloride exchange flux. The K_1/K_m ratio is calculated from eqn. (11) for $K_m = 15$ mM and for $K_m = 50$ mM (for details see text). The calculations are based on the data presented in Table 1 or Fig. 5. The results are given as mean \pm s.e. of mean, the number in brackets being the number of independent experiments. P is the level of significance in a Student's t test, where the K_1/K_m ratio is tested against unity

Inhibitory anion	(n)	$(K_m = 15 \text{ mM})$		$(K_m = 50 \text{ mM})$	
		K_1/K_m	P	K_1/K_m	P
Nitrate	(6)	0.50 ± 0.058	< 0.001	0.45 ± 0.055	< 0.001
Thiocyanate	(5)	0.5 ± 0.10	< 0.02	0.5 ± 0.10	< 0.02
Bromide	(5)	1.4 ± 0.15	< 0.05	1.6 ± 0.23	< 0.05

From eqn. (8) it can be seen that the slope of the curve (α) is equal to

$$(1 - K_m/K_1)/J_{\text{Cl}}^{\text{max}}$$

and the intercept on the ordinate (β) equal to

$$K_m(1 + H/K_1)/J_{\text{Cl}}^{\text{max}}.$$

By elimination of $J_{\text{Cl}}^{\text{max}}$ the following equation is obtained:

$$\frac{K_1}{K_m} = \frac{\beta + \alpha H}{\beta - \alpha K_m}. \quad (11)$$

The value of K_m can be estimated as either 15 mM (Fig. 3A) or 50 mM (Fig. 2A), and Table 2 shows the calculated K_1/K_m ratio corresponding to both these values. It can be seen that the ratio is only slightly influenced by the estimate of K_m . A similar plot using the intracellular (Cl^-) gives nearly the same K_1/K_m ratios (data not shown). The K_1/K_m ratios for Br^- and SCN^- in Table 2 have been calculated (same data as in Table 1) assuming that these anions are also competitive inhibitors of the chloride exchange. The K_1/K_m ratios are significantly different from unity with $K_1 > K_m$ for Br^- and $K_1 < K_m$ for SCN^- and NO_3^- .

Temperature dependence

The chloride steady-state exchange flux was measured in the temperature range from 38 to 7 °C (see Fig. 6). The activation energy (E_A) was found to be 67 ± 6.2 kJ/mole, and was constant in the temperature range studied.

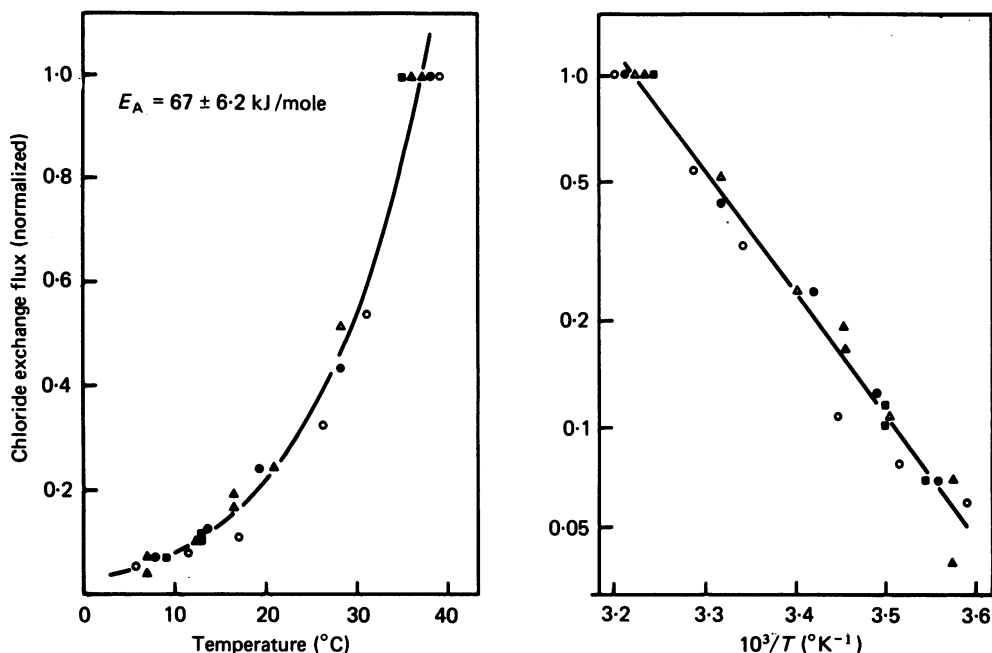


Fig. 6. The temperature dependence of the chloride steady-state exchange flux. The chloride flux at the experimental temperature is given as fraction of the flux measured at 38 °C in the same experiment. The left Figure shows the normalized flux plotted versus the temperature. The equation $J = A \times \exp(-E_A/RT)$ was fitted by an iterative non-linear unweighted least-squares analysis. The continuous line shows the best fit corresponding to $E_A = 67 \pm 6.2$ kJ/mole. The right Figure shows an Arrhenius plot of the log of the chloride flux versus the reciprocal absolute temperature ($1/T$). The line is drawn with a slope corresponding to $E_A = 67$ kJ/mole. The cells were equilibrated at the experimental temperature between 40 min (38 °C) and 100 min (7 °C), before starting the efflux experiments. At 7 °C the cell Na⁺ content increased by about 22 % per hour, and cell K⁺ decreased by about 13 % per hour. At higher temperatures the changes were less. Cell (Cl⁻) changed less than 5 % in all experiments. The measurements are from five separate experiments as indicated.

Membrane potential

Fig. 7 (upper trace) shows a typical oscilloscope trace of a membrane potential recording for an Ehrlich ascites cell in basic Ringer solution with 5 mM-K⁺ and 1 mM-Ca²⁺ at 38 °C. The peak negative value recorded immediately after impalement of the cell is taken to indicate the membrane potential of the unperturbed cell. The values obtained in the present study (-27 ± 1.0 mV ($n = 74$)) correspond to the previously reported values about -24 mV (Lassen *et al.* 1971). Somewhat less negative, stable values (-12 to -14 mV) have been obtained by Smith and co-workers, employing a different technique (Smith & Levinson, 1975; Smith & Adams, 1977), whereas considerably more negative values have been reported based on indirect estimates (Heinz *et al.* 1975, 1977; Burckhardt, 1977).

At increasing external K⁺ concentration, where K⁺ substitutes for Na⁺, the cell membrane depolarizes (see Fig. 8). Similar results were obtained in measurements at

22 °C: $V_m = -23 \pm 0.6$ mV ($n = 166$) with 5 mM- K^+ , -18 ± 1.1 mV ($n = 28$) with 15 mM- K^+ , and -19 ± 1.1 mV ($n = 41$) with 45 mM- K^+ . A depolarization of the cell membrane at high extracellular K^+ was also found in a previous study (Lassen *et al.* 1971), and has recently been reported by Burckhardt (1977) and by Pershadshingh, Johnstone & Laris (1978). In the present study the external Ca^{2+} concentration was 1 mM. In the absence of added Ca^{2+} the membrane potentials have been reported to be less negative (Smith, Mikiten & Levinson, 1972; Smith & Levinson, 1975), and unaffected by external (K^+) (Pershadshingh *et al.* 1978).

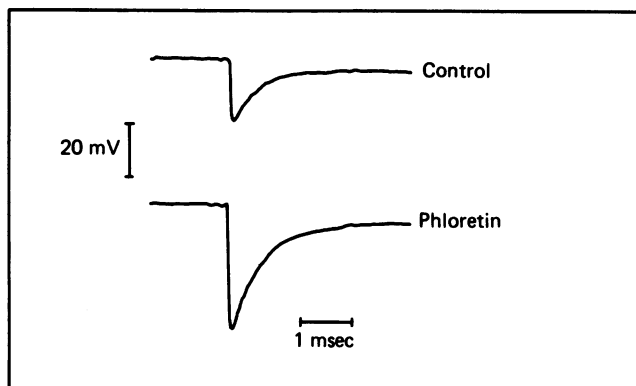


Fig. 7. Typical recording of the membrane potential from an Ehrlich ascites cell at 38 °C in basic Ringer solution (5 mM-external K^+ , 1 mM- Ca^{2+}) (upper trace) and in Ringer solution with phloretin, 0.25 mM, added (lower trace). The peak value recorded immediately after penetration of the cell membrane with the micro-electrode tip is taken to represent the membrane potential, the subsequent decay being ascribed to an electrical leak around the impaling micro-electrode.

Membrane conductance, net (conductive) permeability and apparent permeability to chloride

The membrane potential (V_m) varies with $\log_{10} (K^+)_e$ with a slope of about 21 mV per decade in the region of 5 mM-external (K^+) (see Fig. 8). Assuming a linear relationship near 5 mM-external (K^+) the transference number for K^+ (t_K) can be calculated from the slope (see eqn. (12)), because the measured V_m which may be regarded as the initial value after a sudden change in external K^+ concentration, was found to be reasonably constant with time and because the Nernst potentials for Cl^- (E_{Cl}) and Na^+ (E_{Na}) are essentially constant in this region (see Christoffersen, 1973; Lassen, 1977; Lassen, Pape & Vestergaard-Bogind, 1978):

$$t_K = \frac{\partial V_m}{\partial E_K} = \frac{\partial V_m}{(RT/F) \partial \ln (K^+)_e} = \frac{\partial V_m / \partial \log_{10} (K^+)_e}{0.0617}. \quad (12)$$

From the data shown in Fig. 8 the value of t_K was estimated to be 0.34. In addition, the transference numbers for Cl^- (t_{Cl}) and Na^+ (t_{Na}) could be calculated from the equations:

$$t_K E_K + t_{Na} E_{Na} + t_{Cl} E_{Cl} = V_m \quad (13)$$

$$t_K + t_{Na} + t_{Cl} = 1 \quad (14)$$

The values of E_K , E_{Na} , and E_{Cl} and the calculated transference numbers are given in Table 3. Similar results were obtained in experiments at 22 °C, where the slope of V_m vs. $\log_{10} (K^+)_e$ was about 10 mV per decade and the estimated values of t_K , t_{Na} , and t_{Cl} were 0.17, 0.15, and 0.68, respectively.

In the above calculation we have neglected current carried by ions other than K^+ , Na^+ , and Cl^- (e.g. OH^- , bicarbonate, sulphate and phosphate). A leak conductance

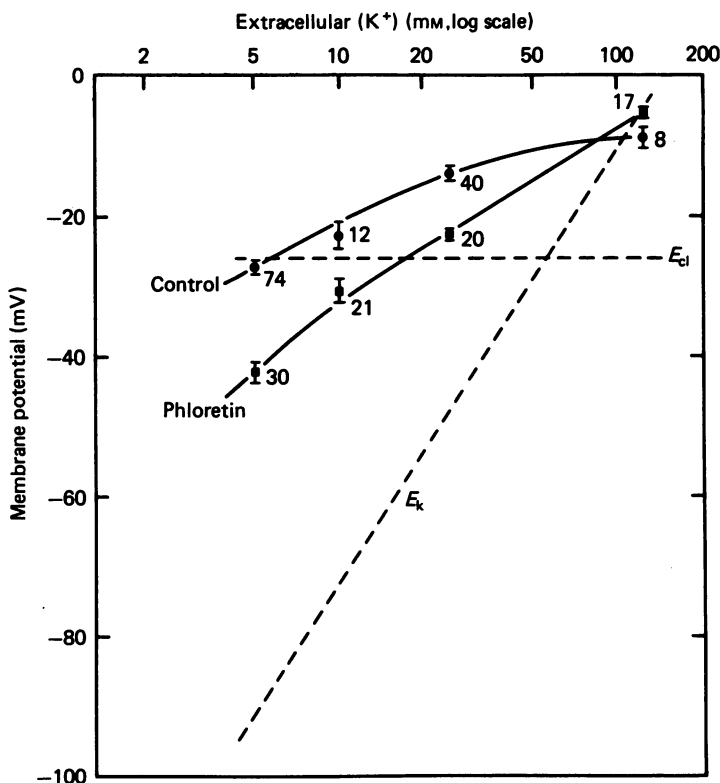


Fig. 8. Dependence of the membrane potential with and without the presence of phloretin on the K^+ concentration of the suspending medium, K^+ being substituted for Na^+ . After preincubation in basic Ringer solution an aliquot of cells was washed and resuspended in medium with the K^+ concentration indicated, with and without the addition of phloretin, 0.25 mM, and ten to twenty potential recordings were obtained from 2 to 12 min after the change in suspending medium. Note log scale on abscissa. The potentials are given as mean values \pm s.e. of mean, the number of measurements being indicated on the Figure. The Nernst potentials for Cl^- (E_{Cl}), and K^+ (E_K) are indicated as dashed lines.

caused by the impalement has not been taken into consideration because the measured potentials (peak values) are taken to indicate the V_m of the unperturbed cell (see Lassen, 1977; Lassen & Rasmussen, 1978). Although there is increasing evidence for the existence of an electrogenic Na^+-K^+ pump in Ehrlich ascites cells (Heinz *et al.* 1975, 1977; Eddy & Philo, 1976), its possible contribution to the over-all membrane potential has been neglected in the present calculations, because the value is rather uncertain, and presumably low in cells in steady-state in ordinary Ringer solution (Laris, Bootman, Pershadsingh & Johnstone, 1978).

At 38 °C the exchange flux of $^{42}\text{K}^+$ in normal Ringer solution was found to be 11.2 ± 0.6 p-mole/cm².sec ($n = 7$). From this value the membrane conductance for K^+ (G_{K}) could be calculated (see Methods). The total membrane conductance (G_{m}), and the membrane conductances for Na^+ (G_{Na}) and Cl^- (G_{Cl}) could then be calculated from the equations:

$$G_{\text{K}} = t_{\text{K}} \times G_{\text{m}} \quad (15a)$$

$$G_{\text{Na}} = t_{\text{Na}} \times G_{\text{m}} \quad (15b)$$

$$G_{\text{Cl}} = t_{\text{Cl}} \times G_{\text{m}} \quad (15c)$$

The calculated values of G_{K} , G_{Na} , and G_{Cl} are given in Table 3. In the above calculations E_{K} , E_{Na} , and E_{Cl} have been calculated from the measured over-all cellular

TABLE 3. Estimate of transference numbers and membrane conductances in Ehrlich ascites cells.

The cells were incubated with 5 mM- K^+ , 1 mM- Ca^{2+} at pH 7.40, 38 °C

	Control	Phloretin*
Membrane potential† (mV)	-27 ± 1.0 (74)	-42 ± 1.5 (30)
Equilibrium potentials‡ (mV)		
E_{K}	-97 ± 0.8 (8)	-97 and -86 ± 0.7 (4)
E_{Na}	43 ± 2.8 (6)	43 and 47 ± 2.8 (4)
E_{Cl}	-23 ± 1.2 (8)	-23 and -30 ± 2.2 (4)
Transference numbers		
t_{K}	0.34	0.57
t_{Na}	0.32	0.35
t_{Cl}	0.34	0.09
Membrane conductances ($\mu\text{S}/\text{cm}^2$)		
G_{K}	14	14–18
G_{Na}	14	9–11
G_{Cl}	14	2–3
G_{m}	42	24–32

* The membrane potentials in the presence of phloretin were recorded immediately after transfer to Ringer solution containing phloretin, 0.25 mM. The cellular ion concentrations were observed to change only slowly with time in the presence of phloretin. The two values of E_{K} , E_{Na} , and E_{Cl} shown in the right column represent the value immediately after exposure to phloretin followed by the value (\pm s.e. of mean) after 50 min preincubation. The range of membrane conductances calculated in the presence of phloretin corresponds to the range of J_{K} (9–12 p-mole/cm².sec, see text).

† Mean \pm s.e. of mean with number of measurements in brackets.

‡ Mean \pm s.e. of mean with number of independent experiments in brackets.

concentrations of K^+ , Na^+ , and Cl^- , assuming equal activity coefficients in cell water and in the extracellular medium, and neglecting the available evidence of intracellular compartmentation of Na^+ and Cl^- (Smith & Adams, 1977), or nuclear sequestration of these ions (Pietrzyk & Heinz, 1974). It can be shown, however, that the calculated values would not be seriously affected by such sequestration. Furthermore, the passive K^+ fluxes have been assumed to be diffusional, although there is evidence of an exchange diffusion component (Mills & Tupper, 1975; Tupper, 1975). This component would appear to be small, however, in cells with high intracellular K^+ concentration, and suspended in ordinary Ringer solutions (see Fig. 6 in Mills & Tupper (1975)).

The permeability constant to K^+ (P_{K}) was calculated from the $^{42}\text{K}^+$ exchange

flux, and P_{Na} from G_{Na} and the corresponding diffusional net flux of Na^+ (see Methods). The conductive (electro-diffusional) unidirectional chloride flux corresponding to the chloride conductance was also calculated, and hence the net (conductive) P_{Cl} . In addition, the apparent P_{Cl} corresponding to the measured unidirectional tracer exchange flux of chloride was calculated (see Methods). The values are given in Tables 4 and 5. The values of P_{K} and P_{Na} correspond reasonably

TABLE 4. Estimate of membrane net permeability to K^+ , Na^+ , and Cl^- in Ehrlich ascites cells. The cells were incubated with 5 mM- K^+ , 1 mM- Ca^{2+} at pH 7.40, 38 °C

Permeability constant	Control (10^{-8} cm/sec)	Phloretin (0.25 mM) (10^{-8} cm/sec)
P_{K} (calc. from tracer exchange flux)	10.4	12-15
P_{Na} (calc. from membrane conductance)	4.4	2.6-3.5
P_{Cl} (calc. from membrane conductance)	3.9	0.6-0.8

TABLE 5. Unidirectional chloride fluxes and estimated membrane permeabilities to chloride (P_{Cl}) in Ehrlich ascites cells. The conductive unidirectional flux and the net (conductive) P_{Cl} are derived from the membrane conductance to chloride (see text). The apparent P_{Cl} is calculated from the measured chloride tracer exchange flux

	Unidirectional chloride flux (p-mole/cm ² .sec)		Chloride permeability (10^{-8} cm/sec)	
	Conductive flux	Tracer exchange flux	Net (conductive) P_{Cl}	Apparent P_{Cl}
Control	4.0	63	3.9	62
Phloretin (0.25 mM)	0.8-1.1	9-16	0.6-0.8	10-20

well to the values reported by Mills & Tupper (1975) based on K^+ and Na^+ tracer flux data. It can be seen (Table 5) that the conductive, unidirectional chloride flux and the net (conductive) P_{Cl} are only about 6% of the tracer exchange flux and the apparent P_{Cl} , respectively.

Effects of phloretin on chloride exchange, membrane potential, and membrane conductance

Phloretin was found to be a potent inhibitor of the chloride steady-state exchange flux which was reduced from 63 ± 4 p-mole/cm².sec ($n = 19$) in control cells to 9-16 p-mole/cm².sec (two expts.) in the presence of phloretin (0.25 mM).

The cell membrane was hyperpolarized in the presence of phloretin. Fig. 7 (lower trace) shows a typical oscilloscope trace of a membrane potential recording from an Ehrlich ascites cell after exposure to phloretin (0.25 mM) in basic Ringer solution at 38 °C. The mean value of the recorded potentials was -42 ± 1.5 mV ($n = 30$; $P < 0.001$ compared to control cells). At increasing external (K^+) the cell membrane was depolarized (see Fig. 8). The slope of V_{m} vs. $\log_{10} (\text{K}^+)_e$ was about 35 mV per decade at 5 mM- K^+ . The calculated values of t_{K} , t_{Na} , and t_{Cl} are given in Table 3. Similar results were obtained in measurements at 22 °C: $V_{\text{m}} = -38 \pm 0.7$ mV ($n = 129$) with 5 mM- K^+ , -28 ± 1.2 mV ($n = 18$) with 15 mM- K^+ , and -17 ± 1.0 mV

($n = 23$) with 45 mM- K^+ . The slope of V_m vs. $\log_{10}(K^+)_e$ was about 22 mV per decade, and t_K , t_{Na} , and t_{Cl} were estimated to be 0.38, 0.19, and 0.42, respectively.

In the presence of phloretin (0.25 mM) the steady-state exchange flux of $^{42}K^+$ at 38 °C was found to be 7.9 ± 1.0 p-mole/cm².sec ($n = 4$), significantly lower than the flux in the absence of phloretin ($P < 0.02$). From the $^{42}K^+$ flux and the transference

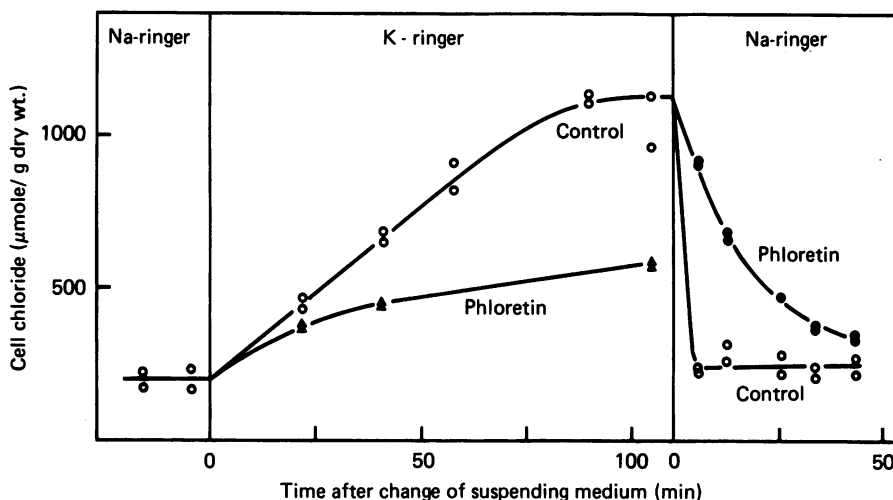


Fig. 9. The increase in cellular chloride content after transfer to K-Ringer and the decrease in cellular chloride content after resuspension in basic Ringer solution (typical experiments). After preincubation in basic Ringer solution (Na-Ringer) for 30 min Ehrlich cells were transferred to K-Ringer (150 mM- K^+ substituted for Na^+), with and without addition of phloretin, 0.25 mM. After 110 min in K-Ringer the control cells were again washed and resuspended in basic Ringer solution with and without addition of phloretin.

numbers the membrane conductances for K^+ , Na^+ , and Cl^- were calculated (see Table 3).

The membrane potentials were measured within few minutes after exposure of the cells to phloretin, whereas the $^{42}K^+$ -exchange flux was measured following 50 min preincubation in the presence of phloretin in order to attain a steady-state ion distribution. From the measured flux P_K was estimated as $12-15 \times 10^{-8}$ cm/sec (cf. eqn. (6)) assuming V_m to be in the range -42 to -30 mV (see Table 3). Assuming a constant P_K during the preincubation, the $^{42}K^+$ exchange flux corresponding to the condition under which the potentials were recorded could then be calculated to $9-12$ p-mole/cm².sec. This corrected $^{42}K^+$ flux value was used in the above calculations.

Phloretin decreased G_{Cl} to about 20% of the control value, whereas G_{Na} was reduced to about 70%, and G_K was slightly increased (see Table 3). In addition, P_K and P_{Na} were calculated in the presence of phloretin (see Methods). P_K was slightly increased and P_{Na} slightly reduced (see Table 4), the ratio P_K/P_{Na} being increased by a factor of 2. In *Amphiuma* giant red cells a similar hyperpolarization, a low anion conductance, and an increased ratio P_K/P_{Na} in the presence of phloretin have been reported by Vestergaard-Bogind & Lassen (1974). Finally, the conductive

unidirectional chloride flux, the net (conductive) P_{Cl} , and the apparent P_{Cl} were calculated (see Methods). The values are given in Table 5.

Fig. 9 shows the net movement of chloride in cells equilibrated in basic Ringer solution (Na-Ringer) after transfer to K-Ringer, and after retransfer to Na-Ringer. It can be seen that in both cases phloretin inhibited the net movement of chloride.

The membrane potential measurements at high external (K⁺) in the presence or absence of phloretin (see Fig. 8) were made within a few minutes after transfer of the cells from basic Ringer solution. The membrane potential was depolarized immediately after transfer to K-Ringer whereas the net uptake of chloride (see Fig. 9) was much slower. This finding implies that V_m was transiently less negative than E_{Cl} and that the chloride distribution transiently deviated from electrochemical equilibrium. This deviation from equilibrium distribution of chloride was also found in the absence of phloretin although it was more pronounced when the net movement of chloride was inhibited by phloretin.

A similar transient deviation from equilibrium distribution of chloride was seen in basic Ringer solution after addition of phloretin. The cell membrane was hyperpolarized to -42 mV immediately after exposure to phloretin (Figs. 7, 8), whereas the net loss of chloride was much slower. Between 50 and 100 min after addition of phloretin, E_{Cl} could be calculated as -30 ± 2.2 mV ($n = 4$). Thus, in more than 50 min E_{Cl} had only decreased from -23 to -30 mV.

DISCUSSION

Saturation kinetics and competitive inhibition of chloride exchange

The experiments in which the external Cl⁻ concentration is varied (Figs. 2, 3) show that the chloride steady-state exchange flux saturates with increasing Cl⁻ concentration, consistent with a saturable carrier model for chloride transport. $K_{\frac{1}{2}}$ was estimated as 50 mM (Fig. 2) or 15 mM (Fig. 3) for extracellular Cl⁻, and 20 mM or 13 mM for intracellular Cl⁻. The relationship between extra- and intracellular $K_{\frac{1}{2}}$ has been discussed by Dalmark (1975). The reasons for the differences between the two saturation curves are as yet unknown, but differences in ionic strength, cell volume, chloride distribution ratio (r_{Cl}), as well as an eventual inhibitory effect of lactate, may play a role. Recent findings show, however, that lactate apparently does not exchange with sulphate (Levinson, C., personal communication), which may be transported by the same mechanism as chloride (Levinson, 1978).

A reduced chloride flux at high Cl⁻ concentrations as seen in Fig. 3 has also been described in nystatin-treated human red cells (Cass & Dalmark, 1973), in which case Cl⁻ was regarded as a non-competitive inhibitor of its own transport (Dalmark, 1976a, b). In agreement with that, Levinson (1978) has proposed a two site model for chloride and sulphate transport in the Ehrlich cell. Such a model could account for the 'self inhibition' at high concentrations seen in Fig. 3.

The chloride flux is inhibited by several other anions as demonstrated in Table 1, and Figs. 4, 5. The K_1/K_m ratios in Table 2 suggest the following sequence of increasing affinity for the chloride transport system: Br⁻ < Cl⁻ < SCN⁻ = NO₃⁻. An inhibitory effect of Br⁻ and NO₃⁻ has previously been reported (Levinson & Villereal,

1976). The sequence of affinity reported above is, however, at variance with previous findings: Levinson & Villereal (1976) found a decreased rate constant for chloride exchange in Br^- media, and in NO_3^- media the rate constant for chloride exchange has previously been found to be unchanged (Grobeck *et al.* 1963; Aull, 1972; Levinson & Villereal, 1976). The data of Aull *et al.* (1977) show, however, in agreement with the present findings, a decreased rate constant for chloride exchange in NO_3^- media. In human red cells the corresponding sequence is: $\text{Cl}^- < \text{Br}^- < \text{NO}_3^- < \text{SCN}^-$ (Dalmark & Wieth, 1972). The inverted sequence of Cl^- and Br^- could indicate that the electrical field strength of a positively charged transport site is higher in Ehrlich cells than in red cells (see e.g. Wright & Diamond, 1977).

In the experiments with chloride exchange in NO_3^- media (Figs. 4, 5) there are no changes in ionic strength, cell water, or r_{Cl} . In this case the chloride steady-state exchange flux can be described by Michaelis-Menten kinetics with NO_3^- as a competitive inhibitor with a higher affinity for the anion transport system than Cl^- . The present results are in agreement with the notion that the anions share a common transport system with a rather low specificity towards the carried anion.

Levinson & Villereal (1976) have previously reported, based on experiments where acetate replaced Cl^- , that 40 % of the chloride transport in Ehrlich cells was carrier mediated and followed a saturation curve, whereas the present data suggest (see below) an exchange component of 95 %. This quantitative difference in the estimate of the exchange component can conceivably be caused by (i) the temperature dependence of the conductive (diffusional) pathway being markedly less than that of the exchange pathway, and/or (ii) acetate interference with the chloride transport.

(i) The experiments of Levinson & Villereal (1976) were made at 20–22 °C. If the temperature dependence were markedly less for the conductive (diffusional) pathway than for the exchange pathway (see below), the conductive pathway might represent only 6 % of the total flux at 38 °C (present data), but a much larger fraction at lower temperatures. The present data do not permit conclusions to be drawn concerning the temperature dependence of the conductance pathway: the transference number for Cl^- was found to be higher at 22 °C than at 38 °C (see Results), but the data shown in Fig. 6 do not support the existence of a component of the chloride flux with a low activation energy.

(ii) Levinson & Villereal (1976) interpreted their data assuming that acetate does not influence chloride transport. Inhibition of chloride exchange by acetate has been observed, however, in Ehrlich cells (Hoffmann *et al.* 1975; Aull *et al.* 1977) and in human red cells where acetate is a non-competitive inhibitor (Gunn *et al.* 1973). We have tried to fit eqn. (10) to the data of Levinson & Villereal (1976, Fig. 4) with the assumption that essentially all the chloride transport is carrier-mediated and that acetate is a non-competitive inhibitor of Cl^- exchange. With a K_m of 20 mM and a K_i of 180 mM the calculated curve seems to fit the data reasonably well.

The presence of an extremely rapid inorganic anion exchange system is well documented in red cells (Gunn *et al.* 1973; Cass & Dalmark, 1973; see Dalmark, 1976*a, b*; Fortes, 1977; Cabantchik, Knauf & Rothstein, 1978), and an anion transport model has been proposed (Wieth, 1972; Gunn, 1972; Dalmark, 1976*a, b*). In the human erythrocyte the number of anion exchange sites has been estimated as 10^6 sites per cell with a turnover rate of 5×10^4 (ions/sec) per site at 38 °C, corresponding to a maximal flux of 5.7×10^{-8} mole/cm².sec (Brahm, 1977). This is three orders of magnitude higher than the value of 63 p-mole/cm².sec found in the Ehrlich cell. The difference can conceivably be caused by a reduced density of anion exchange sites in

Ehrlich cells (corresponding then to about 8000 sites per cell) or by an extraordinarily high turnover rate in red cells which are highly specialized for O₂ and CO₂ transport and for bicarbonate-chloride exchange (the Hamburger shift). In this context it may be noted that the rate of sulphate exchange, which probably is in both cell types mediated by the same transport system as chloride exchange (for references see below), appears to be of similar magnitude in Ehrlich cells (Levinson & Villereal, 1975) and in red cells (Schnell, Gerhardt & Schoeppe-Fredenburg, 1977).

Carrier mediated anion transport in Ehrlich cells has also been demonstrated for inorganic phosphate (Levinson, 1972), lactate (Spencer & Lehninger, 1976), and sulphate (Levinson & Villereal, 1975). The latter is probably transported by the same mechanism as chloride (Villereal & Levinson, 1977; Levinson, 1978), similar to the findings in the human erythrocyte (Schnell *et al.* 1977). Carrier mediated transport of inorganic anions has also been demonstrated in muscle (Harris, 1958; Skydsgaard, 1977) and recently, chloride-bicarbonate exchange has been shown to be involved in the regulation of intracellular pH in nerve and muscle (see Thomas, 1977).

Temperature dependence of chloride exchange

The Arrhenius activation energy reported here (about 67 kJ/mole, constant in the temperature range from 38 to 7 °C) is in reasonable agreement with the findings of Hempling & Kromphardt (1965), who reported a Q_{10} around 3 (corresponding to E_A about 82 kJ/mole) in the temperature range from 37 to 22 °C. Levinson & Villereal (1976) found a smaller temperature dependence from 24 to 4 °C (E_A about 12 kJ/mole). In recent experiments, however, Levinson, C. (personal communication) has reinvestigated the temperature dependence of the chloride transport and obtained an E_A of about 67 kJ/mole. A value of 82 kJ/mole has been reported for human red cells in the temperature range from 15 to 38 °C (Brahm, 1977).

Membrane potential and membrane conductance

The actual magnitude of the membrane potential of Ehrlich ascites cells in ordinary Ringer solution is at present somewhat uncertain. As discussed by Heinz *et al.* (1977) some error apparently is inherent in all the methods used for direct or indirect estimates of the membrane potential in these cells. In this study we have used the values directly recorded by micro-electrode puncture of the cells as a measure of the membrane potential. The values correspond to the calculated E_{Cl} (see Table 3) in accordance with the general assumption of a thermodynamic equilibrium distribution for Cl⁻ across the cell membrane at steady-state (see Lassen *et al.* 1971). The validity of E_{Cl} as an estimate of V_m has been seriously questioned, however: Nuclear sequestration of Cl⁻ (Pietrzyk & Heinz, 1974) or intracellular compartmentation (Smith & Adams, 1977) have been reported, resulting in the true E_{Cl} being either 15–20 mV more negative or about 6 mV more positive than the uncorrected E_{Cl} .

The membrane conductances of Ehrlich cells for K⁺ and Na⁺ calculated in this study (both about 14 μ S/cm², see Table 3) are at variance with the values of 74 and 3 μ S/cm², respectively, reported by Mills & Tupper (1975). If G_K and G_{Na} are calculated from the diffusional K⁺ and Na⁺ fluxes summarized in Fig. 8 in their paper and assuming a membrane potential of -24 mV (see eqn. (4)), it would appear, however, that G_K and G_{Na} are about equal and both of the magnitude 20–30 μ S/cm².

The membrane conductance to chloride is found to be of about the same magnitude as G_K and G_{Na} (see Table 3). This finding is surprising since the rapid tracer exchange of Cl^- (with a time constant of a few minutes) has been generally assumed to imply a high G_{Cl} (see e.g. Mills & Tupper, 1975). If the chloride tracer exchange flux (63 p-mole/cm².sec) were diffusional, the corresponding G_{Cl} (cf. eqn. (5)) would equal 230 $\mu S/cm^2$. For technical reasons direct micro-electrode measurements of the membrane conductance in Ehrlich cells are not available at present. In a previous study (Lassen *et al.* 1971) attempts were made to measure the increment in the resistance seen by the micro-electrode tip after penetration into the cell. However, the apparent specific membrane resistance was only about 70 Ωcm^2 (corresponding to a G_m of about 14 000 $\mu S/cm^2$), and it was concluded that the resistance measurement was dominated by a leak in the cell membrane caused by the impaling micro-electrode.

Table 5 summarizes the conductive unidirectional chloride flux derived from G_{Cl} , the corresponding net (conductive) P_{Cl} , the unidirectional chloride tracer exchange flux, and the corresponding apparent P_{Cl} in control cells and in the presence of phloretin. These data suggest that about 95 % of the chloride transport is mediated by an electrically silent exchange diffusion. A similar finding has recently been reported by Heinz *et al.* (1975, 1977), based on a different experimental approach (similar to that of Hunter (1971, 1977)). Moreover, their estimate of net P_{Cl} (0.057 ml per gram (dry wt.) per minute (Heinz *et al.* 1975), corresponding to about 5×10^{-8} cm/sec) is of similar magnitude to the net P_{Cl} found in the present study. Based on furosemide inhibition of the chloride transport Aull *et al.* (1977) have also concluded that a major part of the Cl^- exchange flux may occur by a one for one exchange. In human red blood cells the net P_{Cl} is about $1-3 \times 10^{-8}$ cm/sec (Dalmark, 1976*b*; Hunter, 1971, 1977; Knauf, Fuhrmann, Rothstein & Rothstein, 1977) or four orders of magnitude less than the apparent P_{Cl} at 38 °C (5×10^{-4} cm/sec, Brahm, 1977). Thus, whereas the chloride exchange in human red cells is three orders of magnitude faster than that in Ehrlich cells, it would appear that the net P_{Cl} is of similar magnitude.

In the presence of phloretin the chloride exchange is reduced to about 20 % of the control value (see Table 5), in accordance with recent findings of Levinson (1978). In red cells phloretin is an extremely potent inhibitor of chloride self-exchange (Wieth, Funder, Gunn & Brahm, 1974). The reduced permeability at 38 °C in the presence of phloretin is, however, likely to be of similar magnitude in Ehrlich cells and in red cells.

In the present study phloretin is also found to inhibit G_{Cl} both at 37 °C (Table 3) and in preliminary experiments at 22 °C. At 37 °C the net (conductive) P_{Cl} is reduced to about 20 % (see Table 5). Phloretin also inhibits the net movement of Cl^- (see Fig. 9), and in the presence of phloretin the Cl^- distribution across the cell membrane may deviate from electrochemical equilibrium (see Results).

The net (conductive) P_{Cl} in Ehrlich cells in the presence of phloretin is estimated as $6-8 \times 10^{-9}$ cm/sec (see Table 5). In egg phosphatidylcholine bilayer vesicles, however, the P_{Cl} (at 38 °C, pH 7.0) can be estimated as about 10^{-9} cm/sec for Cl^- exchange and only about 5×10^{-11} cm/sec for net movement (see Toyoshima & Thompson, 1975). Thus, the ground permeability of the cell membrane (see Lew &

Beaugé, 1979) may be two orders of magnitude lower than even the reduced permeability found in the presence of phloretin. This finding may indicate that the conductance pathway does not represent a simple electrodiffusional leak. A possible relation between anion conductance and exchange pathways in red cells is presently under discussion (Vestergaard-Bogind & Lassen, 1974; Knauf *et al.* 1977; see Cabantchik *et al.* 1978). The finding that phloretin inhibits chloride conductance and exchange to about the same extent in the Ehrlich cell (about 80 %, see Table 5) might indicate that the exchange and conductance pathways are not completely separate and distinct modes of transport, but may involve common elements.

In conclusion, the available evidence would indicate the presence of an anion exchange diffusion system in Ehrlich ascites cells with similar properties to the extremely rapid anion exchange system present in red cells from all origins (Wieth *et al.* 1974).

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